BEHAVIOR OF THE CELL SURFACE DURING CLEAVAGE

III. On the Formation of New Surface in the Eggs of Strongylocentrotus Pulcherrimus[1]

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In the preceding paper of this series (1938), it was shown that the surface of a sea urchin egg cleaving in Ca-free medium is stretched during the process of segmentation to cover the two resulting blastomeres, but that during the interkinetic period, after cleavage has been completed, this previously existing surface is reduced in area, and an apparently new surface is formed in the region involved in the cleavage furrow. This conclusion was drawn from numerous observations that adhering kaolin particles can be found at any point on the surfaces of the two blastomeres at the time when cleavage is completed, but that following this, the particles slowly retreat from the cleavage furrow region, and before the end of interkinesis this area is invariably quite devoid of particles.

Since this conclusion depended solely upon observations of particles attached to the outside of the denuded egg, it was obviously desirable to examine the cortical protoplasm, in order to rule out entirely the possibility that the particles were attached to some investing membrane whose behavior might be different from that of the true protoplasmic surface. An opportunity to settle this point was offered by the method of Motomura (1935), who reported observations on the behavior of pigment granules in the cortex of *Strongylocentrotus pulcherrimus* egg, which, he discovered, could be clearly seen with the aid of a blue filter. His conclusions, however, were considerably at variance with those of the authors, and it therefore became more interesting than ever to combine studies of the the particle-bearing surface layer with those of the granule-containing cortex. The results of this work are presented herewith in three sections. In the first will be presented a description of the pigment granules of *Strongylocentrotus pulcherrimus* eggs as well as some correlated observations on *Arbacia* eggs; the second part will deal with observations on the behavior of the *Strongylocentrotus* granules during cleavage and interkinesis; and the third, with combined observations of particle migration and granule behavior.

[1] This study was assisted in part by a grant from the Foundation for the Promotion of Scientific Research of Japan.

Pigment Granules of Strongylocentrotus, Arbacia pustulosa, and A. punctulata

For the original account of the pigment granules of *Strongylocentrotus pulcherrimus* eggs, Motomura's 1935 paper may be consulted. Briefly, they are small, orange in color, and poorly differentiated from the surrounding protoplasm when viewed with white or yellow light. If, however, a Wratten No. 49 filter is interposed between the eggs and the light source, the granules become more sharply differentiated, appearing dark-red or almost black with the light source used by the authors [2] (*see* Motomura, 1935, Fig. 64). In sections of eggs fixed by chrome-osmium mixtures or by chrome-formol fixatives with later osmication, the granules are strongly blackened.

The position and consequent visibility of these granules vary with the physiological condition and stage of cleavage of the eggs. For this reason, the results of three methods of observation—i.e., of living eggs with white and with filtered light, and of stained sections—have been combined to form the final picture of the distribution of the granules in unfertilized, fertilized and cleaved eggs.

Unfertilized Eggs in White Light.—Examination of the cortical region of living, unfertilized eggs under high magnification with artificial light or with sunlight (Plate I, *A*–1) shows structurally undifferentiated protoplasm of a light orange color extending to the "protoplasmic surface film" (*see* Chambers, 1938).[3] If the focus is raised, the surface of the egg appears to give off an orange color, but this color is diffuse and cannot be definitely attributed to any particular granules.

Unfertilized Eggs in Filtered Light.—If a Wratten No. 49 filter is introduced into the optical system, the egg acquires a just-perceptible, dark-reddish line at the circumference of the largest optical section, and when the focus is raised to bring the upper surface into view, scattered

[2] Osram point-light bulb mounted in a Leitz-Wetzler lamp with a transformer of 6 volts, 5 amperes.

[3] Before Chambers' paper came to their notice, the authors had performed various simple experiments to determine the real existence of this extremely thin, transparent layer which was so consistently found to be present that it was for some time thought to be a refraction artifact. It is visible with direct and dark-field illumination on eggs in sea water and Ca-free medium, and on exovates formed by compressing either fertilized or unfertilized eggs in sea water. On eggs normally fertilized in sea water, the protoplasmic surface film can be distinguished lying beneath the hyaline plasma layer. If such eggs are transferred to Ca-free medium, the hyaline plasma layer dissolves away inside the fertilization membrane, leaving the underlying surface film as the only visible structure closely investing the egg. In only one case was it found possible to effect a change in the appearance of this film. When unfertilized eggs were compressed, and then released before they burst, the protoplasmic surface film on the contracted surface lost its usual perfect contour, appearing roughened and irregular.

granules of this color appear, as nearly as can be determined, immediately below the protoplasmic surface film (Plate I, *B*–1).

Unfertilized Eggs in Section.—Unfertilized eggs treated with OsO_4 show a layer of blackened granules, which are distinguished from other granular inclusions by their slightly larger size, and lie so closely beneath the protoplasmic surface (Plate I, *C*–1) that they appear to be in contact with it.

Fertilized Eggs in White Light.—In less than two minutes after fertilization or after artificial activation, the egg in optical section presents a quite different picture, aside from the appearance of the fertilization membrane and the gradually thickening hyaline plasma layer. In white light, beneath the protoplasmic surface film, a zone of extremely finely

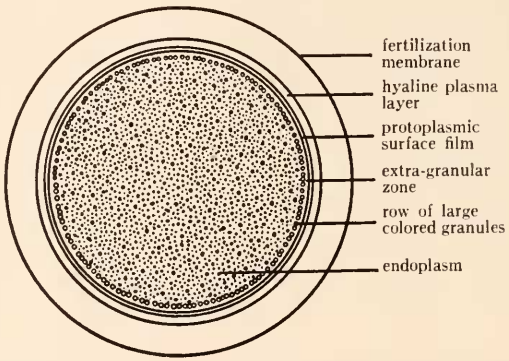

Fig. 1. Diagram of fertilized egg of *Strongylocentrotus pulcherrimus* in sea water.

granular (but not hyaline) protoplasm has appeared, apparently as a result of the centripetal migration, by approximately 1.5 μ, of the coarse granules which had been occupying this region (Fig. 1 and Plate I, *A*–2, *A*–3). This zone, which will hereafter be called the "extra-granular zone," gradually widens, reaching a width of about 2 μ before cleavage begins. Centripetal to the extra-granular zone and forming an outer boundary to the central heterogeneous granular protoplasm is a slightly irregular ring of larger, definitely orange granules, whose color is transmitted to the adjacent extra-granular zone. If the focus is raised, it can be seen that the upper surface no longer gives off the orange radiation which appeared in the unfertilized egg.

Fertilized Eggs in Filtered Light.—When observed with filtered light, the fertilized egg in optical section is seen to have acquired a prominent ring of dark-reddish granules whose position corresponds to that of the orange granules seen with white light (Plate I, *B*–2). At the surface

level, these present the same picture as those in the unfertilized egg, i.e., they lie scattered at random.

Fertilized Eggs in Section.—Osmicated fertilized egg sections show a row of strongly blackened granules at a corresponding distance within the egg cortex [4] (Plate I, *C–2*). On the basis of these observations and others presented below the authors are convinced that the granules which appear as the prominent ring in the fertilized egg move *inward* at fertilization from their previous position immediately inside the protoplasmic film, rather than *outward* from the endoplasm. The fact that their presence in the egg before fertilization can be detected only as a diffuse orange radiation can be explained on the ground that they are lying so closely in contact with the concave surface of the outer protoplasmic surface film as to be individually indistinguishable in optical section.

The conspicuous red granules of the *Arbacia* egg have been objects of interest for many years (Harvey, 1910; McClendon, 1910, et al.). It has generally been accepted that these granules move from the interior of the egg to the cortex at the time of fertilization, but this observation is difficult to prove. However, it is certain that a demonstrable rearrangement takes place during the four and a half minutes following fertilization in that the granules, which in the unfertilized egg appear to be located at various depths from the surface, move into a single layer in the now firmly gelated cortex (Plate II, *B, C*). This behavior is observed in the eggs of both *Arbacia punctulata* of Woods Hole and *A. pustulosa* of Naples. Although the details of the *Arbacia* granule movement are decidedly at variance with those which have been observed in *Strongylocentrotus*, the possibility seems to be worth considering that the rearrangement may be caused by inward migration, as is the case in *Strongylocentrotus*, rather than by the outward movement generally regarded as the sole process involved. This same change in distribution occurs after artificial activation.

Pigment which can be extracted from the tests of sea urchins is generally known as "echinochrome" after MacMunn's nomenclature, and it has been proved that the pigment is an oxidation-reduction dye (MacMunn, 1885, 1889; Griffiths, 1892; Cannan, 1927). Pigment which is contained in the eggs of several kinds of sea urchins including *Arbacia* ("arbacine" of Vlès and Vellinger, 1928) has also been identified as echinochrome (McClendon, 1912; Ball, 1934), and the observation that the granules move to the cell periphery after fertilization concurrently with a great increase in respiration lent further support to the

[4] Because of the shrinkage of the eggs incident to fixing and sectioning as well as the difficulty of determining the exact level of section in a given case, and since the distances in question are very small, the making of precise measurement was rejected as involving too large an error to have any meaning.

conclusion that they perform a respiratory function (Runnström, 1928). Crude trials were made to determine whether observable changes take place either in the orange coloration or in the spectroscopic absorption

PLATE I

A. Strongylocentrotus eggs in white light.

A–1. Unfertilized egg. Notice that there is no differentiated structure inside the egg.

A–2. Fertilized egg in Ca-free artificial sea water. Fertilization membrane has been removed mechanically and hyaline plasma layer has dissolved away. Note extra-granular zone around periphery and row of granules inside it. Dark appearance of granules is due rather to high refractivity than to intense color. Protoplasmic surface film is not clearly defined in this photograph.

A–3. Fertilized egg in normal sea water, showing fertilization membrane, hyaline plasma layer, protoplasmic surface film, extra-granular zone and row of granules (cf. diagram, Fig. 1).

B. Strongylocentrotus eggs in filtered light—mixed culture of fertilized and unfertilized eggs in Ca-free medium. (Since sea-urchin eggs cannot be fertilized in Ca-free medium, there was no danger of contamination of unfertilized eggs by excess sperm from fertilized eggs. As extra precaution, eggs were well washed after fertilization and allowed to stand some time before being mixed with unfertilized eggs.) In order to insure easy differentiation between fertilized and unfertilized eggs, fertilization membranes were not removed, but were left in place in collapsed condition which results upon their introduction into Ca-free medium. This collapse of fertilization membrane permitted simultaneous focusing upon fertilized and unfertilized eggs, which is impossible when membranes are normally expanded. Aside from collapse of membrane and disappearance of hyaline plasma layer, appearance of eggs in sea water and Ca-free medium is identical.

B–1. Unfertilized egg, showing absence of differentiation in granular cytoplasm.

B–2. Fertilized egg, showing dark-red ring. (Outermost structure is collapsed fertilization membrane, beneath which protoplasmic surface film is poorly visible at right side. Hyaline plasma layer has dissolved away in Ca-free medium.)

C. Strongylocentrotus eggs in sections.

C–1. Unfertilized egg showing row of osmium-blackened granules closely applied to cell surface.

C–2. Fertilized egg with extra-granular zone and row of granules beneath it.

D. Strongylocentrotus egg in late interkinesis—Ca-free preparation showing difference between "old" surface on polar side of blastomeres provided with extra-granular zone, and new surface along cleavage furrow without extra-granular zone. Note high refractivity of newly-formed surface.

E. Strongylocentrotus egg in four-cell stage (Ca-free medium). Note that "old" surface appears only on outer sides of blastomeres.

F. Strongylocentrotus egg in section. Separated blastomere in which polar and furrow regions are clearly differentiated by contour, showing absence of extra-granular zone and presence of highly refractive membrane in furrow region.

G. Section of spherical separated blastomere of *Strongylocentrotus* in which direction of preceding cleavage is indicated by differentiated structures as in *F.*

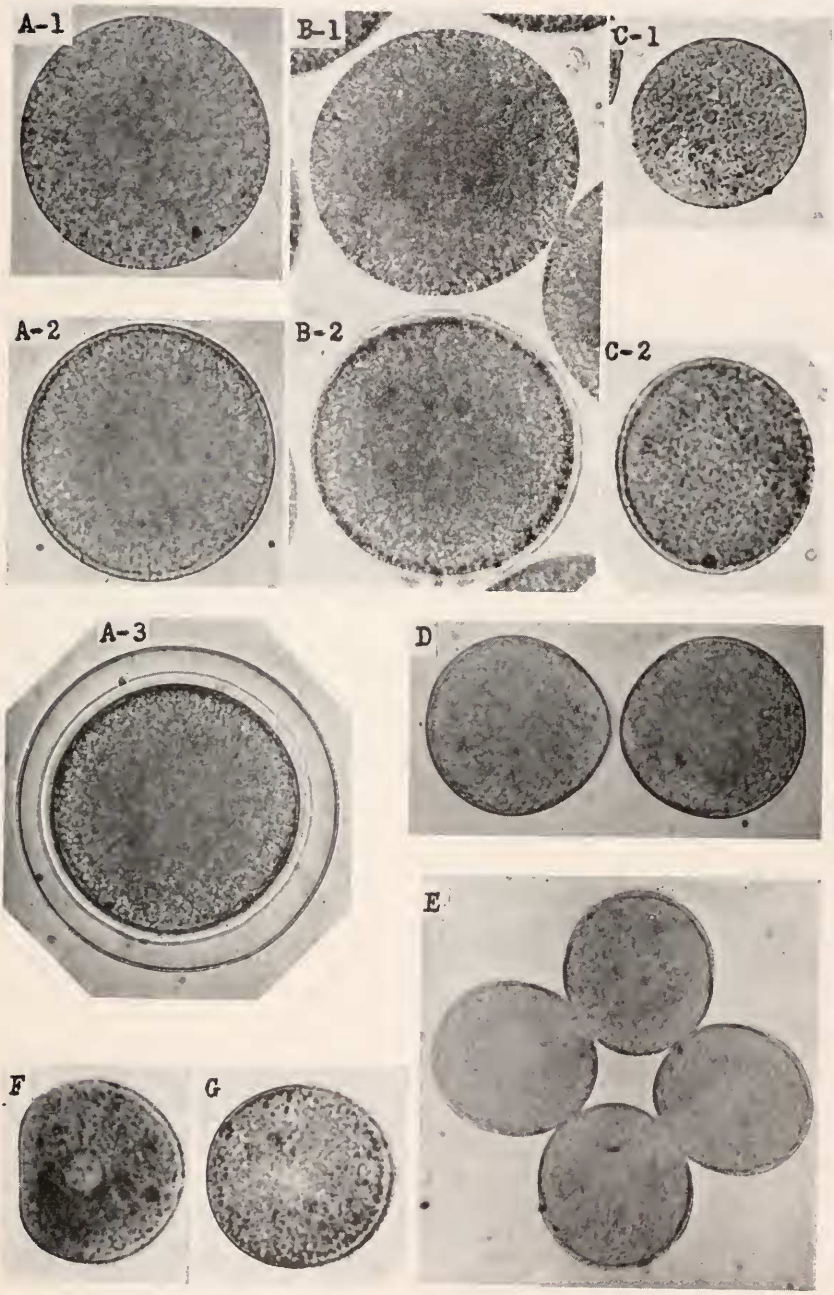

PLATE I

of the *Strongylocentrotus* granules under the influence of oxidants or reductants. Although it is still premature to make a final conclusion, at least in water extract and acetone extract of the pigment and in the pigment *in vivo*, no change could so far be detected, contrary to the case of the pigment extracted from the tests of this species (Suto, 1938).

A further marked difference in physiological function between these granules and those of *Arbacia* is found in the fact that these granules do not break down upon cytolysis or liberation into sea water, while the *Arbacia* granules do (Heilbrunn, 1928). However, the somewhat similar position and the fact that there is a remarkable change in distribution following fertilization suggest that there may be a closer relationship between them than is apparent from the existing data.

Behavior of the Cortical Elements in Cleavage

In this section, because of its importance in the later discussion, the behavior of the cortical elements in eggs cleaving in Ca-free artificial sea water will be described in detail.

The eggs of *Strongylocentrotus pulcherrimus* were prepared for observation by the following procedure: insemination was performed in a minimum of sea water, and when the fertilization membranes were par-

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PLATE II

A. Consecutive photographs of division of *Strongylocentrotus* egg taken with direct sunlight through filter at low magnification, later enlarged. This series aims especially to show interrelation of dark-red ring and kaolin particles. (Room temperature 13° C., process plates used for photographing.)

A–1. Two kaolin particles stuck on sides of furrow (indicated by arrows). Note that dark-red ring completely covers surface.

A–2. One minute later. Furrow has deepened and particles are carried further into it.

A–3. Two minutes, thirty seconds later (than first photograph). Cleavage is complete. Note that dark granules are accumulating around point of last connection between blastomeres.

A–4. Three minutes, thirty seconds later. Blastomeres have rounded up.

A–5. Five minutes later. Dispersion of granules and migration of kaolin particles out of furrow have started simultaneously.

A–6. Six minutes later. First sign of opening of granular ring appears.

A–7. Seven minutes later.

A–8. Ten minutes later. New surface has fully developed. Note that kaolin particles are well outside furrow and dark granules are distributed only on polar sides of blastomeres.

B. *Arbacia pustulosa*, unfertilized egg in dark-field illumination. Surface view of egg, showing that pigment granules are at different depths from surface, so that simultaneous clear focus is not obtainable. (Note that this is reverse of case with *Strongylocentrotus*, in which granules are in single layer closely applied to protoplasmic surface film.)

C. Surface view of fertilized egg of *A. pustulosa;* pigment granules are now in a single layer.

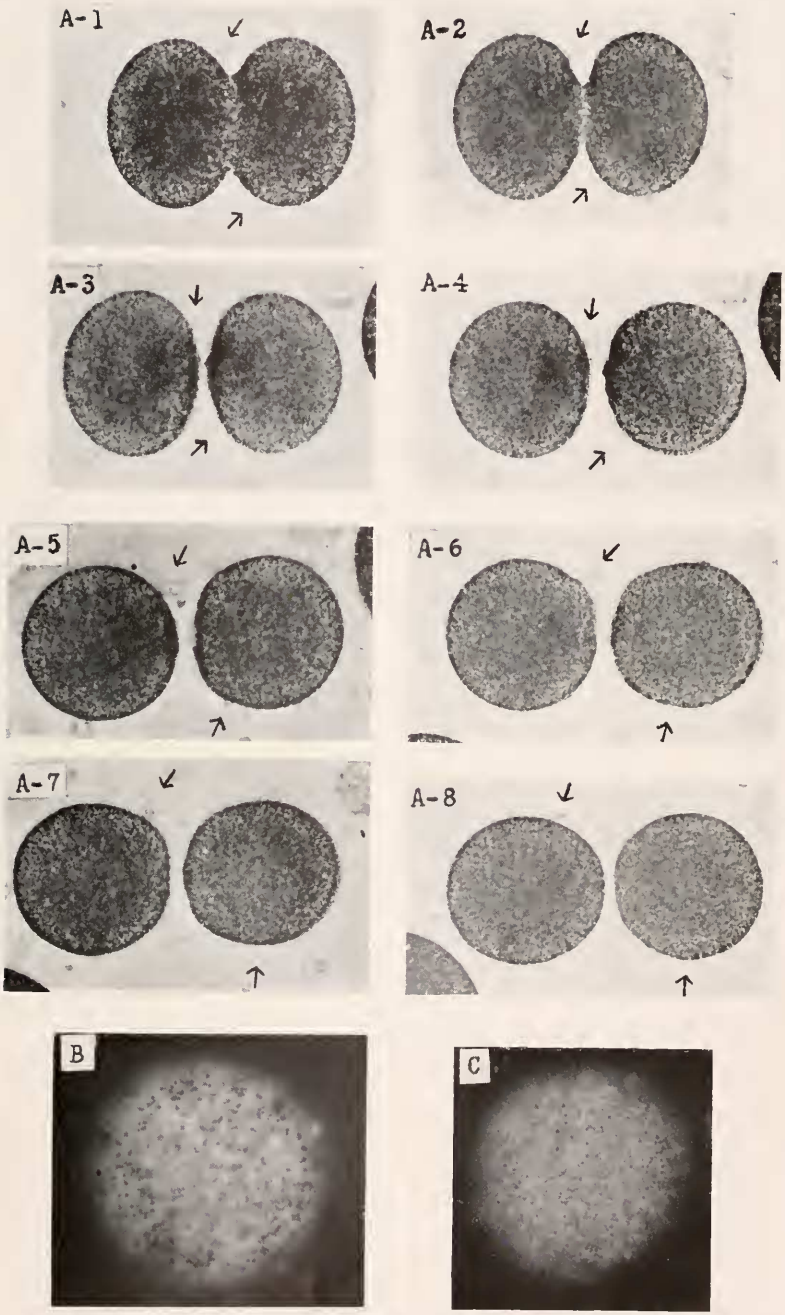

PLATE II

tially raised (about 1 minute later), a mixture of equal parts of Ca-free artificial sea water and 0.53M (isotonic) KCl was added to the eggs. In this solution, the membranes became abnormally expanded and fragile, and were removed without injury to most of the eggs by the use of a fine pipette of the proper bore. Since too long a stay in the presence of an excess of KCl produces abnormalities in the eggs at the time of cleavage, they were returned to Ca-free sea water, washed repeatedly, and allowed to develop until just before the first cleavage. At this time, an equal amount of isotonic KCl was again added to the suspension (since it was found that, in this species, in Ca-free sea water alone the blastomeres tend to come into close contact during interkinesis, rendering observation difficult, while this does not occur if excess KCl is present, although in all other respects the cleavage picture is the same.)

In White Light.—As cleavage begins in *Strongylocentrotus* eggs, the mutual relations of the granular ring, the extra-granular zone and the protoplasmic surface film are maintained practically unchanged. However, as the furrow progressively deepens, the extra-granular zone at the polar regions [5] becomes noticeably narrower, presumably because of stretching (Fig. 2, *A*, *B*, *C*). About the time when the separation of the blastomeres is complete, the granules appear to accumulate to some extent in the cortical protoplasm forming the walls of the furrow, and, moreover, are not so uniformly equidistant from the surface in this region (Fig. 2, *D*). During the first part of the interkinetic period, the accumulation of granules in the walls of the furrows is gradually dispersed, and the extra-granular zone in the furrow region, which has become continuous by the complete severance of the blastomeres, is gradually opened, starting from this central point (Fig. 2, *D*, *E*). As it opens, its place is occupied by an increasing extent of surface which is remarkably similar to that of the unfertilized egg; i.e., no extra-granular zone is present, the orange granules are not separately distinguishable and the coarse granular endoplasm extends all the way to the surface film (Fig. 2, *E*, *F*). However, this outer membrane is sharply defined against the surrounding medium because of a high refractive index which is found only in this newly-formed surface, and is not characteristic of the polar surfaces of the blastomeres (Plate I, *D*) or of the unfertilized egg surface (Plate I, *A*–1).

In the second cleavage, a practically similar process is repeated, the formation of new surface taking place in such a way that the extra-granular zone comes to lie only on the outer surfaces of the four blasto-

[5] In the papers of this series, the authors have adopted the terminology of Chambers with respect to the topography of cleaving eggs; i.e., the furrow is referred to as the equator in defining the poles. (See diagrams in previous papers.)

meres (Plate I, *E*); and so far as the authors can determine, this is regularly kept up until the blastula stage (see Motomura, 1935, Fig. 71).

In Filtered Light.—In the cleaving egg seen with filtered light, the dark-red ring covers the entire surface (Plate II, *A*–1, *A*–2). When

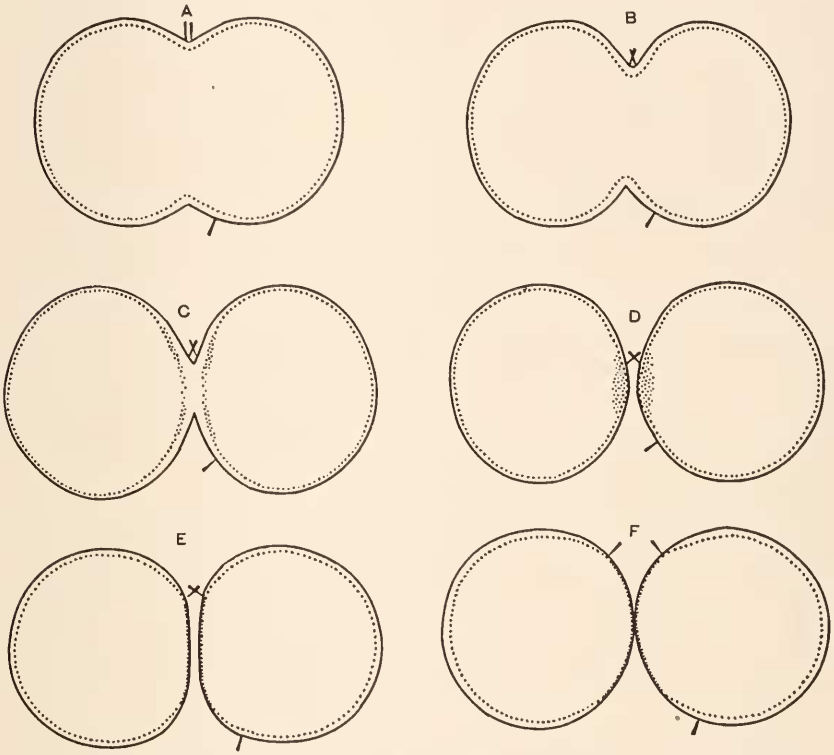

FIG. 2. Diagram of typical *Strongylocentrotus* experiment to show, simultaneously, behavior of kaolin particles adhering to surface, extra-granular zone and granular layer. (Changing outline of cleaving egg and positions of adhering particles are traced from photographs. Positions of granules are indicated by dots.)

A. Cleaving egg; kaolin particles are situated in furrow region; extra-granular zone has narrowed at poles, presumably because of stretching.

B. Kaolin particles are carried inward by deepening of furrow.

C. Extra-granular zone in furrow region widens, at poles becomes extremely narrow.

D. Extra-granular zone regains uniform width around blastomeres; granules are accumulated at point of last connection between blastomeres.

E. Formation in furrow region of new surface, lacking in extra-granular zone. Kaolin particles have been pushed out of furrow; accumulated granules have dispersed—they are now lined up in close proximity to newly-formed surface.

F. Late interkinetic stage; extra-granular zone over polar two-thirds of blastomeres has widened as though released from tension; upper particles are now well outside of furrow, at boundary between old and new surfaces.

the severance of the blastomeres is nearly completed, the dark-red ring in optical section becomes very thick at the sides of the furrow (Plate II, *A*–3, *A*–4); after cleavage is completed, it first regains a more uniform width all around the blastomeres (Plate II, *A*–5) and then breaks at the hyaline connecting stalk (Plate II, *A*–6, *A*–7) and becomes less and less a complete ring, finally including only about 65 per cent of the circumference of the blastomeres (Plate II, *A*–8). The surface where the ring has opened is characterized by the absence of the red granular ring, as is the case with unfertilized eggs. In the four-cell stage, the dark-red granules are distributed on the outer sides of the four blastomeres, corresponding exactly to the distribution of the extra-granular zone as seen in white light.

Sections.—In sections of eggs fixed with osmium mixtures, however, contrary to the expectation from observations in filtered light, there is a row of blackened granules extending completely around both the newly separated blastomeres and blastomeres in the interkinetic period. In the early stages of cleavage, at the poles, the ring is closer to the surface —i.e., the extra-granular zone is narrower than in the other regions; while in the cortex adjoining the furrow, the granules are less perfectly aligned, sometimes lying 5 or 6 μ inside the surface layer. In the later interkinetic stages, the ring of granules extends around the polar part of the blastomere section at a uniform distance from the surface as in the egg before cleavage. Around the periphery on the furrow side, this separating zone of protoplasm (extra-granular zone) is quite lacking, and the row of blackened granules is found closely applied to the surface membrane, as in unfertilized eggs. However, in this case, the granules appear to be more numerous, forming a continuous, highly refractive line (Plate I, *F, G*). This greater accumulation of granules in fertilized eggs than in unfertilized eggs may explain why this part of the blastomere surface is more clearly defined, in the living condition, than that of the unfertilized eggs.

When fertilized eggs are allowed to cleave in sea water, so that the fertilization membrane and hyaline plasma layer are intact and the blastomeres closely in contact as soon as the cleavage furrow is complete, it is impossible to distinguish either orange granules or dark-red ring along the contact surface (Motomura, Fig. 70). However, studies of sections reveal that, as is the case when cleavage takes place in Ca-free sea water, the extra-granular zone covers the entire surface before the complete severance of the two blastomeres, but that later it retreats from the furrow region while the row of granules is always encircling the endoplasm. From this, it must be concluded that the apparent absence of orange color in white light and of the dark-red ring in filtered light is

controlled not by the actual absence of the granules but rather by the optical conditions imposed by the presence of the extra-granular zone or by the spatial relations of the blastomeres.

Another indication that the apparent absence of the dark-red granules in the walls of the cleavage furrow in sea water is an optical illusion arising from the very close association of the two surfaces is furnished by the observation that when two fertilized eggs with perfectly complete rings of dark-red granules are brought into contact, the granules appear to be absent from the parts of the egg peripheries which are in contact. Therefore, since the visibility of the dark-red ring with a filter appears to be largely dependent upon the optical conditions, it must be concluded that one cannot safely determine the presence or absence of granules on the sole basis of observations with the filter.

Summarizing this section, it can be said that direct observation of the cortex reveals, as was predicted in the previous paper from the behavior of the kaolin particles, that the existing surface of the uncleaved egg is pulled into the furrow during the process of segmentation, and that only when the interkinetic period is reached, does a new surface begin to be formed along the furrow sides.

Simultaneous Observation of Cortical Elements and Kaolin Particles Affixed to the Surface of Strongylocentrotus Eggs

Kaolin particles were added to a Ca-free culture of the eggs, prepared in the way described above. After thorough mixing, excess particles were removed by washing. Eggs were mounted in a deep hollow slide, a cover glass was fastened in place by means of vaseline, and the whole preparation was immersed in a larger volume of Ca-free medium to reduce local heating by the light source.

When photographs were taken with filtered light, in order to make the exposure time as short as possible, direct sunlight and a low magnification were used. An egg with suitably located particles was selected and photographed at intervals from before the completion of cleavage until nearly the end of the interkinetic period. Figures shown in Plate II are enlarged pictures of such a series. In these photographs, a clear image of the extra-granular zone was sacrificed in order to show the dark-red ring.

In these series, eggs were chosen with particles at the head of the cleavage furrow, because this is the spot where it is anticipated that the new surface will begin to appear. If so, the particles which are at the head of the furrow are expected to come to lie at the border between the old and new surfaces when the latter is formed. In filtered light, this border will be the place where, in optical section, the dark-red ends. In

white light, such particles will be found where the extra-granular zone ends. This expectation has been borne out perfectly by many observations.

The unfailing coincidence between the position of such kaolin particles and the border line between the old and new surfaces (*see* diagram, Fig. 2, *E, F*) leads to the conclusion that the behavior of the kaolin particles can be taken as a direct indication of the behavior of the cortical material. Evidently, the kaolin particles must be adhering to the protoplasmic surface film, which is the outermost structure persisting in a Ca-free medium, and the above findings show that the protoplasmic surface film does not slip over the underlying cortical layer as does the hyaline plasma layer [6] (Dan, Yanagita and Sugiyama, 1937).

Discussion

In the preceding sections the observations have been presented in the light of the authors' interpretations. However, since Motomura's interpretations sometimes differ from those of the authors, a few words will be devoted to comparing the two.

In connection with cleaved eggs in sea water, Motomura concluded that the granules are present only along the free surfaces of the blastomeres and that they are absent along the contact surfaces,—a conclusion based on the fact that the dark-red granules in filtered light can be seen in the former position but not in the latter. So far as the observation is concerned, our finding coincides perfectly with Motomura's. However, if the identity of the dark-red granules and the osmium-stained peripheral granules in sections is assumed, as the authors are compelled to do from the coincidence in size, position and behavior between them, it must be concluded that the granules are present all over the surface at any time, and that it is the extra-granular zone which is present only on the free surfaces and not along the newly-formed surfaces (Motomura, Fig. 70). The authors' explanation of the observation is that the granules will take a dark-red color in filtered light anywhere and at any time, but when they are closely in contact with a surface, it is not possible to distinguish them. However, both sets of conclusions agree in saying that the part of the surface of cleaved eggs in which the dark-red granules are invisible in filtered light is a newly-formed surface.

Concerning the case in which eggs are kept in a Ca-free medium and are allowed to cleave in it, Motomura came to a rather confusing conclu-

[6] It incidentally follows that a new protoplasmic film must be produced over the newly formed surface of the blastomeres. This is to be expected from the previously reported observations, in which no intact protoplasmic surface could be found without the investing film.

sion. Quoting him: "In short, the new cell boundaries are not formed in the calcium-free sea water in the course of the early cleavages. The surface of each blastomere is covered by the extension of the pigmented cortical cytoplasm. But, when the blastomere is replaced in the normal sea water, it regains the capacity of forming the new cell boundaries. In this case the blastomere is able to form the pluteus. When the egg is cultured successively in calcium-free sea water, no formation of the embryo in it is possible. In this case the distribution of the pigment granules is irregular" (p. 239). From this description, he apparently missed the formation of new surface in Ca-free sea water entirely. In the authors' experience, in order to observe the formation of new surface, special precautions are necessary to secure a good condition of the cultures. Overheating of the preparation by the light source or a slight shortage in the quantity of the culture medium at the time of observation immediately impairs the eggs. During the first cleavage, when such injury occurs, the eggs invariably stop their development at the stage in which the separation of the blastomeres is just completed and the granules are accumulated at the sides of the furrow (Plate II, *A*–3, *A*–4). Therefore, even as early as the beginning of the interkinetic period, it is possible to predict whether or not an egg under observation will undergo further cleavages. Cultures were encountered, especially toward the end of the season, in which all the eggs remained in this state. In such eggs, the granules sooner or later begin to clump together irregularly, and the eggs finally die. But so far as the authors are aware, as long as the condition of the egg remains favorable, each cleavage is regularly followed by the formation of new surface. Hence, it is impossible to accept Motomura's argument in the same chapter, that the failure of larvae in Ca-free sea water to develop into plutei is due more to the lack of new surface formation than to the falling apart of the blastomeres.

The assumption that the granules under discussion are located only in the cortical layer and not in the endoplasm has already been made by Motomura on the ground that exovates do not contain the granules. This conclusion is tenable only under the circumstance that the granules do not break down when the cells are injured, unlike the case with *Arbacia* granules. The authors' further experiments with this view in mind, together with their study of sectioned eggs, support Motomura's contention.

The authors are happy to acknowledge their indebtedness to the Marine Biological Laboratory of Woods Hole and to the Stazione Zoologica of Naples for the use of their research facilities.

Summary

1. In *Strongylocentrotus pulcherrimus* eggs, the granules seen in the living egg as orange-colored in white light and dark-red in light filtered through a Wratten No. 49 filter, and the large blackened granules seen in osmium-fixed sections, have been shown to be identical on the basis of size, position and behavior.

2. In unfertilized eggs, these granules are located directly beneath the surface, in which case it is difficult to distinguish them individually.

3. On fertilization, these granules migrate inward from the surface by 1.5 μ, leaving a finely granular zone of the same width at the cell periphery. This zone is called the " extra-granular zone " in the present paper.

4. During the process of segmentation, the equatorial surface of the uncleaved egg with the extra-granular zone and the granular layer forms the cleavage furrow without change in the mutual relations of the component parts except for the fact that the extra-granular zone on the polar side becomes thinner and the granules become more numerous on the sides of the furrow.

5. After the completion of cleavage, the accumulation of granules in the furrow disappears and the extra-granular zone on the polar side regains its pre-cleavage width—i.e., the distribution of the granules around the blastomeres momentarily becomes uniform.

6. The above stage is immediately followed by a phase of formation of new surface along the furrow region in which the extra-granular zone retreats from the sides of the furrow and the granules come into direct contact with the protoplasmic surface film. This arrangement on the new surface is the same as that of the unfertilized egg.

7. Kaolin particles adhering to the cell surface at the head of the cleavage furrow later come to lie at the border between the old surface (provided with the extra-granular zone) and the new surface (which lacks this zone). This is taken as an indication that the new surface is formed from the tip of the furrow, and that the protoplasmic surface film which is carrying the kaolin particles does not slip over the underlying cortex.

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